Reconsolidation and extinction are dissociable and mutually exclusive processes: behavioral and molecular evidence

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Reconsolidation and Extinction are Dissociable and Mutually Exclusive Processes: Behavioural and Molecular Evidence

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ABSTRACT

Memory persistence is critically influenced by retrieval. In rats, a single presentation of a conditioned fear stimulus induces memory reconsolidation and fear memory persistence, while repeated fear cue presentations result in loss of fear through extinction. These two opposite behavioural outcomes are operationally linked by the number of cue presentations at memory retrieval. However, the behavioural properties and mechanistic determinants of the transition have not yet been explored; in particular whether reconsolidation and extinction processes co-exist, or are mutually exclusive depending on the exposure to non-reinforced retrieval events. We characterised both behaviourally and molecularly the transition from reconsolidation to extinction of conditioned fear and show that an increase in calcineurin (CaN) in the basolateral amygdala (BLA) supports the shift from fear-maintenance to fear-inhibition. Gradually increasing the extent of retrieval induces a gradual decrease in freezing responses to the CS and a gradual increase in amygdala CaN. This newly synthesized CaN is required for the extinction, but not reconsolidation, of conditioned fear. During the transition from reconsolidation to extinction we established an insensitive state of the fear memory where NMDA-type glutamate receptor agonist and antagonist drugs are unable to neither modulate CaN levels in the BLA nor alter reconsolidation or extinction processes. Taken together, our data indicate both that reconsolidation and extinction are mutually exclusive processes and also reveal the presence of a transition or 'limbo' state of the original memory between these two alternative outcomes of fear memory retrieval, when neither process is engaged.

INTRODUCTION

Persistence or inhibition of aversive and appetitive memories in the brain is determined by retrieval conditions. Following fear conditioning, in which an association forms between a conditioned stimulus (CS) and a footshock (unconditioned stimulus, US), later presentations of the CS can elicit the conditioned fear response of freezing (Blanchard and Blanchard, 1969). Fully consolidated fear memories, when reactivated by brief CS exposure, return to a labile state that is sensitive to disruption by amnestic agents such as protein synthesis inhibitor. From this labilized state the fear memory requires reconsolidation in order to persist in the brain (Nader et al., 2000; Lee et al., 2006). However, if the non-reinforced CS is presented repeatedly, the behavioural outcome is fear memory extinction (Pavlov, 1927) which is characterized by inhibition of the freezing response to the CS and is also protein synthesis-dependent (Vianna et al., 2001). After extinction, the original fear memory is not erased, but is inhibited by the newly acquired extinction memory (Bouton, 1991). These data illustrate the paradox that persistence of fear through reconsolidation and the loss of fear through extinction are both induced by retrieval of the memory (Eisenberg et al., 2003; Pedreira and Maldonado, 2003). To date, the majority of research has focused on understanding the mechanisms responsible for the two extreme outcomes of retrieval: reconsolidation and extinction (Suzuki et al., 2004; de la Fuente et al., 2011). But it is not known how increasing numbers of CS presentations lead from termination of reconsolidation (and protection of the original memory) to extinction. The amygdala plays a critical role in persistence of conditioned fear (Dunn and Everitt, 1988; Campeau and Davis, 1995; LeDoux, 2000). Rapid transitions between states of high and low fear can be induced by a switch in the activation of two distinct subpopulations of neurons in the basolateral amygdala (BLA) (Herry et al., 2008), which is therefore a key locus for the transition between persistence and loss of fear.

While the activities of specific kinases exert a positive influence to increase synaptic efficacy (Sweatt, 2004), specific phosphatases exert a negative influence either to reduce (during long-term depression)

or constrain increases (during long-term potentiation) in synaptic efficacy (Mulkey et al., 1994; Ikegami et al., 1996). As proposed for the formation of the original memory (Malleret et al., 2001), the balance between kinases and phosphatases could be pivotal in the establishment of the dominant memory process engaged by different retrieval events.

The objective of the present study was to investigate the role of the extracellular signal-regulated kinase (ERK) and the Ca^{2+}- and calmodulin-dependent protein phosphatase, calcineurin (CaN) in the BLA on the persistence or inhibition of cued fear memory, and to define the mechanism by which the gradual increase in non-reinforced presentations of the CS shapes the behavioural and molecular transitions between reconsolidation and extinction. We hypothesised that in the amygdala there would be an NMDAR-dependent specific balance between pERK1/2 and CaN levels related to each memory process that underlies the transition from reconsolidation to extinction of fear memory.

MATERIALS AND METHODS

Animals. Adult male Lister-Hooded rats weighing 250-300 g (Charles River) were used. All animals were kept under a 12 h light/dark cycle (lights off at 7 am) and provided with food and water *ad libitum*. All animal procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

Surgeries. Rats were anaesthetized with ketamine hydrochloride (100 mg/kg; Ketaset, Fort Dodge Animal Health) and xylazine (9 mg/kg; Rompun, Bayer), and implanted with 22-gauge stainless steel bilateral indwelling guide cannulae (Plastics One) aimed at the BLA. The coordinates were 3.6 mm posterior to bregma, 4.5 mm lateral to the mid line and 3.6 mm ventral to *dura mater*. Stainless-steel obturators were inserted to maintain patency during recovery and in between infusions.

Intracranial microinfusions. Infusions were carried out using a syringe pump connected to injectors (28 gauge, projecting 4 mm beyond the guide cannulae) by polyethylene tubing. Prior to behavioural testing, animals were habituated to the infusion procedure by the administration of 0.5 μl of sterile saline solution per side (0.25 μl/min). Desalted and phosphorothioate end-capped 18-mer sequence oligodeoxynucleotides (ODNs, Sigma-Aldrich) were resuspended in sterile saline solution to a concentration of 10 nmol of each sequence per μl. The sequences of the antisense oligos targeting the A subunits of Calcineurin were obtained from previous reports (Garver et al., 1999): Calcineurin A1 antisense, 5'-CTC GGA CAT CTC CAG TCA-3'; A2 antisense, 5'-CTC CGG GGC GGC CAT GCT-3' (the underlined codons correspond to the translation initiation complementary site). ODNs with the same composition of bases but in a scrambled order were used: A1 scrambled, 5'-GTC GCA GAT CCT CCA ACT-3'; A2 scrambled, 5'-GCT CGT TAG CCG GCG CGC-3'. Using a basic local alignment search tool (BLAST (Altschul et al., 1997)) the antisense ODNs sequences showed a significant full alignment only for the respective rat mRNA sequences while the scrambled ODNs failed to fully align with any sequence in the database. 0.5 μl of ODN solution per side (0.25 μl/min) was infused 150 min before the first CS of the reactivation or extinction session. The use of short length ODNs, with only three phosphorothioated bases per end and more than one scrambled control sequence significantly reduces the possibility of both non-target (other mRNA sequences) and off-target effects (no Watson-Crick pairs).

Behavioural procedures. Animals were initially individually habituated to the conditioning box (Paul Fray Ltd, UK) for 2 hours. On the training day, rats were placed in the box and after 25 minutes received an auditory conditioned stimulus (CS) presentation (60s clicker, 10Hz, 80 dB) that was coterminous with the presentation of a scrambled footshock (US, 0.5 mA, 0.5 sec) delivered through the grid floor. The training session consisted of two CS-US presentations with an intertrial interval (ITI) of 5 minutes. Twenty four hours later the rats were returned to the box and presented with 1, 4, 7, or 10 CS presentations, ITI = 1min. Depending on the experiment, 24 or 96 hr later animals were again returned

to the conditioning box and presented with one CS. All training, CS presentation and test sessions were video recorded for off line behavioural analysis. The percentage of time freezing (absence of movement except for breathing) during the 1 minute prior to and during the 1 minute CS was manually scored from the videos by an observer blind to the treatment. Statistical analyses were performed using one-way or repeated measures ANOVA, and post hoc comparisons were made using Tukey's test.

Drug injection. NMDAR partial agonist D-Cycloserine (DCS) and antagonist MK-801 (Sigma-Aldrich) solutions were both freshly prepared with sterile saline for intraperitoneal injection (1 ml/kg). The doses of 15 mg/kg for DCS and 0.1 mg/Kg for MK-801 were selected on the basis of their facilitatory (DCS) and inhibitory (MK-801) effects on reconsolidation and extinction of fear memory (Lee et al., 2006). The injections of DCS, MK-801 or saline were given 30 minutes before the CS presentation sessions. Intra-BLA infusions of DCS induce similar effects in memory as the systemic administrations (Walker et al., 2002; Lee et al., 2006; Mao et al., 2006), strongly suggesting that the BLA is one of the main targets of the systemic manipulations of NMDAR used in memory paradigms.

Protein extraction and western blotting. After CS presentation sessions or 24 hours after training (Ctr groups) rats were sacrificed by carbon dioxide inhalation followed by neck dislocation. The brains were rapidly removed and snap frozen on dry ice prior to storage at -80°C. Samples from the BLA were micro dissected using a 0.99 mm in diameter punching tool from 150 μm thick brain sections. To obtain cytoplasmic protein extracts, BLA tissue from each animal was individually disrupted with a dounce tissue grinder (loose pestle, Wheaton) in 100 μl of buffer (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, Pepstatin A 1 μg/ml; Leupeptin 10 μg/ml; PMSF 0.5 mM; Aprotinin 10 μg/ml) and centrifuged at 1000*g* for 15 minutes at 4°C. The supernatant (cytoplasmic protein extract) was transferred to a clean tube and store at -80°C. The protein content was determined by Bradford assay. 5-10 μg of cytoplasmic proteins were separated using a 10% SDS-polyacrylamide gel electrophoresis

(PAGE) and electro-transferred into a nitrocellulose membrane. Blots were probed with: mouse anti-Calcineurin (#610260, BD Biosciences, 1: 2500), mouse anti-ERK1/2 (#610124, BD Biosciences, 1: 5000), rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr 202/ Tyr 204) (D13.14.4E) (Cell Signaling Tech, 1: 500), mouse anti-β-actin [AC-15] (AbCam, 1: 50000), goat anti-rabbit-HRP (Sigma, 1: 10000) and rabbit anti-mouse-HRP (Sigma, 1: 25000) diluted in Tris-buffered saline solution containing 0.1% of Tween-20. A chemiluminescent signal was induced using an enhanced chemiluminescent reagent (Amersham) and images were captured using a cooled CCD camera (ChemiDoc-It, UVP). Signal analysis and quantification was performed using ImageJ software (v1.47a, NIH). Each primary antibody working concentration was adjusted to deliver a linear relationship between the amounts of loaded protein in the blot versus signal intensity. The optical density (OD) of the bands of interest was measured, and the intensity of the experimental conditions (relative OD, ROD) was calculated normalizing these values to the mean OD of the control group. To control for loading variations, the amount of β-actin for each sample was used as a normalizing value. Data were analysed using a one-way ANOVA with Dunnett's test as *post hoc* comparisons.

RESULTS

pERK1/2 and calcineurin are differentially associated with the reconsolidation and extinction of fear memory

According to our previous data (Lee et al., 2006) varying the number of non-reinforced CS presentations in animals with fully consolidated auditory fear memory results in the engagement of either reconsolidation or extinction processes. In the first set of experiments we analysed the effect of two protocols of unreinforced CS presentation on the freezing response to the fear CS and on the levels of two memory-related proteins, pERK1/2 and CaN.

Two groups of rats were fear conditioned by pairing two presentations of a clicker (CS) with a mild electric footshock (US). As expected, during the training session, the percentage of time spent freezing to the CS significantly increased after the first pairing ($F_{(1, 14)} = 122.48$, $p < 0.001$, $\eta^2 = 0.89$; Figure 1A), with no difference between groups ($F_{(1, 14)} = 1.01$, $p = 0.33$). The following day, each group of animals was presented either with 1 or 10 CS (groups 1CS or 10CS, inter trial interval (ITI) = 1 min). Both groups showed fear memory retention as indicated by a high percentage of time freezing to the auditory CS (preCS vs. CS1, $F_{(1, 14)} = 299.91$, $p < 0.001$, $\eta^2 = 0.95$), with no differences between groups ($F < 1$). In the 10CS group, freezing significantly decreased on presentation of the CSs ($F_{(9, 63)} = 10.52$, $p < 0.001$, $\eta^2 = 0.60$). Twenty four hours later a long-term memory test (LTM-TS) was conducted to measure freezing to the CS in both groups. The 10CS group showed a significant reduction in the percentage of time spent freezing compared to the 1CS group ($F_{(1, 14)} = 18.04$, $p < 0.001$, $\eta^2 = 0.56$). Given the well-established finding that NMDAR antagonism during fear memory reactivation induced by a single CS presentation results in subsequent amnesia (Figure 1B and (Lee et al., 2006)), the present findings confirmed that following CS-shock fear conditioning, the presentation of 1 CS results in reconsolidation and thereby

maintenance of the fear response to the CS, while 10 presentations of the CS results in extinction and inhibition of the fear response.

We then analysed the effect of 1 or 10 presentations of the fear CS on the levels of pERK1/2 and CaN in the BLA. Twenty four hours after fear conditioning, separate groups of rats were exposed to 1 or 10 CS as before, or remained in the home cage (non-reactivated control group; Ctr), and sacrificed after 20 or 60 minutes (Figure 1C). BLA cytoplasmic proteins were prepared and analysed by western blots. Other time points after the presentation of 1 or 10 CS were analysed but there was no increase in either pERK1/2 or CaN in BLA cytosolic or nuclear protein extracts (data not shown). The Ctr group used here allowed us to define basal pERK1/2 or CaN levels in BLA 24 hours after training, but before any putative change induced by CS presentations. While pERK1/2 levels were increased after both 1 ($p < 0.01$) or 10 ($p < 0.05$; one-way ANOVA, $F_{(2, 21)} = 5.06$, $p < 0.05$, $\eta^2 = 0.33$) CS presentations compared to the control group (Figure 1D), CaN levels increased only after the presentation of 10 CS ($p < 0.01$, vs. Ctr; one-way ANOVA, $F_{(2, 21)} = 4.77$, $p < 0.05$, $\eta^2 = 0.31$; Figure 1E).

Thus, while the increase in pERK1/2 in the BLA is a common correlate of both reconsolidation and extinction of fear memory, CaN levels are increased only when an extinction memory is being consolidated. This lack of specific association of the pERK1/2 signal with either reconsolidation or extinction indicates that this particular signaling pathway is not a molecular candidate subserving the transition between these memory processes. By contrast, the increase in CaN levels in the BLA emerges as a distinctive and specific characteristic of extinction memory consolidation, and therefore may be required for the shift from maintenance to loss of fear responses that follows increasing numbers of presentations of the fear CS.

High calcineurin levels in the BLA induced by repeated CS presentations are required for consolidation of fear extinction memory

In the next set of experiments we tested the hypothesis of a causal relationship between the increase in CaN levels in the BLA induced by 10 CS presentations and the establishment of a long-lasting extinction memory. We hypothesized that the increase in synthesis of CaN in the BLA is required for the consolidation of the extinction memory, but is not required for reconsolidation of the original fear memory. An antisense oligodeoxynucleotide (ASO) was infused into the BLA to prevent the increase in CaN levels induced by the presentation of 10 CS in animals with a fully consolidated fear memory. This enabled the evaluation of the role of CaN in the consolidation of a fear extinction memory. We also predicted that knockdown of CaN would have no impact on the reconsolidation process.

BLA-cannulated rats were trained with the presentation of two pairings of CS and footshock US, with all animals showing a higher level of freezing to the second CS compared to the first ($F_{(1, 34)} = 216.08$, $p < 0.001$, $\eta^2 = 0.86$; Figure 3A) and with no differences between groups ($F < 1$). The following day the rats received an infusion into the BLA (Figure 2) of either scrambled oligodeoxynucleotides (Scr) or CaN ASO two and a half hours before the presentation of 1 or 10 CS. Even though the freezing levels were somewhat but equally reduced by the administration of either the Scr or CaN ASO into the BLA (Figure 3A) all groups showed retention of fear memory indicated by the high level of freezing to the first CS ($F_{(1, 34)} = 25.86$, $p < 0.001$, $\eta^2 = 0.43$), with no differences between groups ($F < 1$). Within-session extinction by presentation of 10 CS was identified in both ASO and Scr groups which showed a significant decrease in freezing ($F_{(9, 153)} = 7.13$, $p < 0.001$, $\eta^2 = 0.30$; Figure 3A, Re-exposure), with no differences between groups ($F < 1$). To allow for the complete clearance of the oligodeoxynucleotide from the BLA, the long-term memory test (LTM-TS) was conducted 4 days later. At LTM-TS a two-way ANOVA revealed a significant effect of CS ($F_{(1, 34)} = 9.65$, $p < 0.005$, $\eta^2 = 0.22$) no effect of drug ($F_{(1, 34)} = 3.31$, $p = 0.078$) but

importantly there was a significant interaction (CS x Drug: $F_{(1, 34)} = 9.65$, $p < 0.005$, $\eta^2 = 0.22$). Simple main effects analysis revealed a significant differences between 10CS groups treated with Scr and ASO ($F_{(1, 18)} = 16.92$, $p < 0.005$, $\eta^2 = 0.48$; Figure 3A, LTM-TS) but no differences between the 1CS groups ($F < 1$; Figure 3A, LTM-TS).

To confirm that the infusion of CaN ASO into the BLA prevented the increase in the level of phosphatase induced by the extinction protocol, animals were trained with 2 CS-US pairings (effect of CS-US, $F_{(1, 7)} = 25.86$, $p < 0.001$, $\eta^2 = 0.79$; no effect of group, $F < 1$) and 24 hours later, divided into two groups that received an infusion into the BLA of either the ASO or Scr solutions, followed 2.5 hours later by the presentation of 10 CS (Figure 3B). As in the previous experiment, even though the freezing levels were partly reduced by the administration of either the Scr or CaN ASO into the BLA, both groups nevertheless showed good retention of the fear memory ($F_{(1, 7)} = 15.89$, $p < 0.01$, $\eta^2 = 0.69$), with no differences between Scr and ASO groups ($F_{(1, 7)} = 1.87$, $p = 0.21$) and a within-session decrease in freezing ($F_{(9, 63)} = 4.50$, $p < 0.001$, $\eta^2 = 0.39$) that again did not differ between groups ($F < 1$; Figure 3B). One hour after the extinction protocol, the animals were sacrificed and cytoplasmic proteins extracts from the BLA prepared. Western blot analysis revealed a lower level of CaN in the ASO group compared to the Scr group ($p < 0.05$; Figure 3B, bar graph), confirming that the micro infusion of the CaN ASO into the BLA prevented the synthesis *de novo* of the phosphatase induced by the extinction protocol.

The use of ODN to knock down the expression of a particular protein has the potential to produce non-target effects. We therefore used an experimental design that also measured levels of an unrelated protein (β-actin; Figure 2B) following infusion of CaN ASO in the BLA to assess its selectivity for CaN and reducing the likelihood of a non-target effect. The results strongly support a specific effect of the ASO on CaN and hence on memory extinction.

Previous reports show that while BLA inactivation following infusion of GABA(A) receptor agonist muscimol during extinction training prevents fear expression and extinction learning (Laurent et al., 2008), the AMPA receptor antagonist LY293558 infused into the BLA prevents expression of fear, but do not prevent labilization and restabilisation of the fear memory (Milton et al., 2013). Although ODN microinfusion into the BLA before retrieval partially affected fear expression, the effect of CaN knock down on memory extinction cannot be attributed to general BLA inactivation during CS presentations, since both the Scr and ASO groups showed markedly and significantly different levels of freezing at LTM-TS, with the Scr group showing complete fear extinction. Together, these data indicate that specifically blocking the increase in CaN levels in the BLA induced by the 10 CS extinction protocol completely prevented the consolidation of the fear extinction memory, as well as showing that the increase in CaN could not simply have reflected the mere presentation of an auditory stimulus having no association with the fearful US. Given the lack of any drug or CS-drug interaction between Scr and ASO groups under the 10 CS protocol, it can be concluded that CaN knockdown into the BLA did not affect extinction acquisition. Furthermore, the reconsolidation process induced by 1 CS presentation was unaffected by CaN knockdown.

Gradual increases in the number of fear CS presentations result in a "dose-dependent" transition from fear maintenance to fear inhibition

In order to further specify the behavioural and molecular characteristics of the transition from reconsolidation-inducing to extinction-inducing conditions, we analysed the effect of increasing the number of CS presentations in animals with a fully consolidated fear memory on both conditioned freezing and levels of CaN in the BLA.

Two pairings of a clicker CS and shock resulted as before in a significant increase in freezing on subsequent presentations of the CS ($F_{(1, 28)} = 324.11$, $p < 0.001$, $\eta^2 = 0.92$) with no differences between groups ($F_{(3, 28)} = 1.84$, $p = 0.163$; Figure 4A). On the following day animals in each of the four groups were presented with 1, 4, 7 or 10 CS (ITI = 1 min). All groups showed fear memory retention, with a high level of freezing to the first CS ($F_{(1, 28)} = 477.07$, $p < 0.001$, $\eta^2 = 0.95$) with no differences between groups ($F < 1$). There was a decrease in freezing to the CS in the 4 ($F_{(3, 21)} = 7.43$, $p < 0.001$, $\eta^2 = 0.51$), 7 ($F_{(6, 42)} = 10.72$, $p < 0.001$, $\eta^2 = 0.61$) and 10 ($F_{(9, 63)} = 15.00$, $p < 0.001$, $\eta^2 = 0.68$) CS groups (Figure 4A, Re-exposure). Twenty four hours later, freezing was measured following the presentation of 1 CS (LTM-TS). Freezing decreased proportionally in relation with the degree of CS exposure ($F_{(3, 28)} = 6.11$, $p < 0.005$, $\eta^2 = 0.39$). Thus, further *post hoc* analysis revealed that the level of freezing at LTM-TS in the 1CS group was similar to the 4CS group ($p = 0.32$) but significantly higher than in the 7 and 10 CS groups ($p < 0.05$ and $p < 0.005$, respectively), while the level of freezing of the 10CS group was not different from 4 or 7CS groups ($p = 0.1$ and $p = 0.52$, respectively) (Figure 4A). A positive correlation was observed between the level of freezing at the last CS of the re-exposure session and the level of freezing at LTM-TS ($r_s = 0.710$, $p < 0.01$, Figure 4B).

To analyse the effect of the number of CS presentations at retrieval on levels of CaN in the BLA additional groups of fear conditioned animals were presented with 1, 4, 7 or 10 CSs and sacrificed 1 hour later (Figure 4C). To control for the level of CaN in the BLA as a consequence of the consolidation of the original fear memory, the experiment included a control group that was also conditioned, but remained in the home cage until sacrifice 24 hours later. As expected, there was an effect of the number of CSs presented during training ($F_{(1, 50)} = 357.98$, $p < 0.001$, $\eta^2 = 0.88$), with no differences between groups ($F < 1$). The following day, all the CS-exposed groups showed fear memory retention ($F_{(1, 35)} = 444.26$, $p < 0.001$, $\eta^2 = 0.93$), with no differences between groups ($F < 1$, Figure 4C, CS1). ANOVA revealed an effect of group on CaN levels in the BLA ($F_{(4, 50)} = 7.23$, $p < 0.001$, $\eta^2 = 0.37$; Figure 4C, bar graph). *Post hoc*

analysis showed that CaN levels were higher and significantly different from controls only in the 10CS group ($p < 0.001$). A more detailed analysis at the individual level revealed a negative correlation between the level of freezing at the last CS of the exposure session and the level of CaN in the BLA ($r_s = -0.325$, $p < 0.05$, Figure 4D).

These data revealed a monotonic relationship between the number of CS presentations during the re-exposure session and the level of freezing at test, suggesting a gradual behavioural transition between reconsolidation-like to extinction-like mechanisms induced by repeated non-reinforced CS presentations that is negatively correlated with a progressive increase in CaN in the BLA.

NMDAR activity manipulation both modulates calcineurin levels in the BLA and alters the transition from reconsolidation to extinction

In order better to understand the behavioural and molecular characteristics of the memory states that are transitional between reconsolidation and extinction, we took advantage of the bidirectional effects on these memory processes of NMDAR agonism and antagonism (Baker and Azorlosa, 1996; Walker et al., 2002; Suzuki et al., 2004; Lee et al., 2006). Using systemic administration of the NMDAR partial agonist, D-cycloserine (DCS), and the antagonist, MK-801, it was possible to reveal the dominant memory process in operation as well as the engagement of CaN synthesis induced by increasing the number of CS presentations at retrieval.

In accordance with our previous results (Lee et al., 2006), we expected to find bidirectional modulation of the level of freezing at LTM-TS with the administration of the NMDAR partial agonist DCS or antagonist MK-801. We predicted that DCS would enhance or maintain high levels of freezing if administered in conjunction with a 1 CS (reconsolidation) protocol, while MK-801 should have an

amnestic effect, inducing a low level of freezing at test. By contrast, if administered in conjunction with a 10 CS (extinction) protocol, DCS should result in low levels of freezing at LTM-TS by enhancing extinction while MK-801 should prevent extinction and result in a high level of freezing at test. However, the main objective of this experiment was to evaluate the impact of manipulating NMDAR activity at the molecular and behavioural level on the transitional states between reconsolidation and extinction, i.e. the 4 and 7 CS groups, in comparison with the expected effects of the treatments on 1 and 10 CS groups.

Rats were fear conditioned as before with two CS-US pairings. The following day, the animals received an intraperitoneal injection of saline, DCS (15 mg/Kg) or MK-801 (0.1 mg/Kg) and 30 minutes later were exposed to either 1, 4, 7 or 10 CS protocols (Figure 5A). As predicted, the groups exposed to 1 CS showed a main effect of drug at LTM-TS ($F_{(2, 25)} = 5.86$, $p < 0.01$, $\eta^2 = 0.32$), with the MK-801 group showing a lower level of freezing than the saline group ($p < 0.05$; Figure 5B bar graph), while in the 10CS groups, there was a main effect of drug ($F_{(2, 24)} = 18.41$, $p < 0.01$, $\eta^2 = 0.61$) with the MK-801 group showing a higher level of freezing compared to saline ($p < 0.01$; Figure 5E bar graph). The 7CS groups showed a main effect of drug ($F_{(2, 22)} = 18.23$, $p < 0.01$, $\eta^2 = 0.62$) with the MK-801 and DCS groups presenting a higher ($p < 0.05$) and lower ($p < 0.01$) level of freezing than the saline group respectively (Figure 5D bar graph). The 4CS groups showed no differences in the level of freezing at LTM-TS ($F < 1$; Figure 5C bar graph). Taken together, these data both confirm the utility of this approach in revealing the dominant memory process engaged by the different intermediate retrieval conditions, and also highlight the remarkable insensitivity of the 4 CS-induced memory state to bidirectional manipulation of NMDAR activity.

To evaluate the effect of manipulating NMDAR activity on CaN levels in the BLA induced by increasing presentations of the fear CS (Figure 4C), animals were trained as before and the following day they received an IP injection of either DCS or MK-801 30 minutes before the presentation of 1, 4, 7 or 10 CS,

or were returned to the home cage (Ctr). One hour after the exposure session the animals were sacrificed and extracts of BLA cytoplasmic proteins prepared. Western blots revealed that treatment with MK-801 prevented the increase in CaN levels in the BLA observed after increasing the number of CS presentations ($F < 1$; Figure 6A, bar graph). Thus, systemic MK-801 prevented both memory extinction and CaN level increase in the BLA induced by increasing the number of CS presentations. On the contrary, western blots did reveal differences in CaN levels in DCS-treated groups ($F_{(4, 49)} = 3.61$, $p < 0.05$, $\eta^2 = 0.24$; Figure 6B, bar graph). *Post hoc* analysis revealed that in both 7 and 10CS groups there was a higher level of CaN compared to control ($p < 0.05$ in both), showing that the facilitatory effect of DCS on partial extinction induced by 7 CSs is associated with a specific enhancement of the synthesis of CaN in the BLA.

Non-injected animals showed a significant increase in CaN in the BLA only after 10 CS presentations when compared with non-reactivated controls (Figure 4). In order to analyse the effect of NMDAR activity manipulations on the modulation of CaN levels by increasing the number of CS presentations, we injected all groups with the NMDAR partial agonist DCS or the antagonist MK-801, and then compared the effect of CS presentations on CaN levels in the BLA with respect to their non-reactivated, DCS or MK-801 control group. This design thereby enabled us to quantify the interaction between memory retrieval and bidirectional NMDAR activity manipulations.

Therefore, NMDAR activity manipulation affected the 7CS-induced transitional state in a way similar in molecular and behavioural terms to the 10CS protocol, suggesting that 7 CS presentations predominantly engage extinction-like mechanisms. In contrast, NMDAR manipulations did not affect the molecular and behavioural properties of the transitional state induced by the 4CS protocol, strongly suggesting that neither reconsolidation- nor extinction-like mechanisms are engaged. Taken together, these results show that increasing or decreasing NMDAR activity modulates both the behavioural

transition of a fear memory from reconsolidation to extinction and also the engagement of CaN synthesis in the BLA.

DISCUSSION

This study demonstrates that reconsolidation and extinction of a fear CS memory are molecularly distinct and mutually exclusive processes. While the level of CaN in the BLA was increased by the non-reinforced presentation of 10 CSs (that resulted in fear extinction), but not by reactivation of fear by a single CS presentation (resulting in reconsolidation); pERK1/2 was increased in the BLA in both conditions. Preventing the increase in CaN levels in the BLA induced by 10 CS presentations by infusing a CaN antisense oligodeoxynucleotide specifically prevented the consolidation of the extinction memory that depends, therefore, on the upregulation of cytosolic CaN. On the contrary, CaN knockdown in the BLA during 1 CS presentation had no effect on fear memory reconsolidation. Moreover, repeated non-reinforced cue presentations induce a gradual behavioural transition between maintenance and inhibition of fear that was negatively correlated with a progressive increase in CaN in the BLA. Modulation of NMDAR activity differentially affected the levels of CaN in the BLA and concomitantly affected the transition between reconsolidation and extinction induced by increasing the number of CS presentations. The NMDAR antagonist MK-801 prevented extinction and also the increase in CaN in the BLA induced by the strong extinction protocol (10 CS), while the NMDAR partial agonist DCS enhanced fear extinction and induced a significant increase in CaN levels by the presentation of the partial extinction protocol (7 CS presentations). Thus, the increase in CaN synthesis in the BLA mediated by increased NMDAR activity is causally involved in the engagement of extinction mechanisms that result in the long-term loss of fear responding to the CS. At the behavioural level, NMDAR activity bidirectional manipulations showed that some of the transitional states between reconsolidation- to extinction-inducing retrieval conditions do not show a dominant memory process. In the continuum of possible retrieval conditions, reconsolidation and extinction processes are mutually exclusive, separated by an

insensitive phase were the amount of CS exposure terminates the labilization of the original memory, but are insufficient to trigger the formation of the extinction memory.

Role of calcineurin in the persistence of the extinction memory

Fully consolidated cued fear memories, if not retrieved, can persist for up to the entire life of an animal (Gale et al., 2004). If retrieved, reconsolidation or extinction of the original memory may occur depending on the degree of CS exposure (Eisenberg et al., 2003; Pedreira and Maldonado, 2003). There are partially overlapping molecular mechanisms underlying fear memory consolidation, reconsolidation and extinction. For example, each process depends upon *de novo* protein synthesis and NMDAR activation (Davis and Squire, 1984; Miserendino et al., 1990; Nader et al., 2000; Vianna et al., 2001; Lee et al., 2006). However, reconsolidation and consolidation are also dissociable at the molecular level (Lee et al., 2004). Here we have shown that ERK1/2 activation in the BLA occurs during both reconsolidation and extinction, consistent with data showing that ERK1/2 activity is required for both processes (Duvarci et al., 2005; Herry et al., 2006). By contrast, CaN affects consolidation of both the original and the extinction memory in completely opposite ways. Inhibiting or reducing the level of CaN during training of the original memory facilitates fear memory consolidation (Ikegami and Inokuchi, 2000; Baumgartel et al., 2008), suggesting that CaN can modulate the *establishment* of aversive memories. Here we show for the first time that knocking down CaN during reconsolidation had no effect on the original memory, suggesting that the *updating* process induced by memory reactivation does not require CaN, which is instead involved in establishing the fear memory (Ikegami and Inokuchi, 2000). In contrast, CaN knockdown in the BLA during extinction training specifically inhibited the *consolidation of extinction* of the aversive memory without affecting either the expression of fear or within-session extinction (Figure

3A). It is of note that intra-BLA infusion of calcineurin activity inhibitors FK-506 and cypermethrin before extinction have been shown to prevent the acquisition of an extinction memory (Lin et al., 2003).

Thus the extinction protocol has a dual effect on CaN in the BLA: an increase in activity is required either for acquisition of the extinction memory or short-term extinction (Lin et al., 2003); but increased synthesis of new CaN is specifically required for the establishment of a persistent extinction memory (Figure 1 and 3). *In vitro* studies have further shown that inhibition of CaN facilitates LTP (Ikegami et al., 1996) but inhibits the establishment of LTD (Mulkey et al., 1994). Taken together, these findings reveal distinctive molecular mechanisms underlying the formation and maintenance of excitatory and inhibitory memory traces. In the BLA, ERK1/2 activation may account for the maintenance of the excitatory component at reconsolidation, but also the formation of the new inhibitory trace during extinction, while CaN levels may govern the transition from reconsolidation to extinction, possibly by reducing the activation of the original memory trace in response to the CS.

Discrete subpopulations of amygdala neurons have been shown to encode either fear conditioning or extinction memories (Herry et al., 2008). After training, a CS presentation induces the activation of fear neurons, while repeated CS presentations results in the activation of extinction neurons and the inhibition of fear neurons (Herry et al., 2008). Our findings show that while reconsolidation induced by one CS is associated with potentiation-like mechanisms (e.g. ERK1/2 activation), extinction involves both potentiation- and depotentiation-associated mechanisms within the BLA. These data therefore emphasize the central role of the BLA in the maintenance or inhibition of fear, and the unique molecular landscapes associated with reconsolidation and extinction processes.

Molecular and behavioural aspects of the transition from reconsolidation to extinction

In the present study we have revealed novel behavioural and molecular features of the transition between reconsolidation and extinction. Operationally, the use of stepwise increments in the number of discrete CS presentation events at retrieval provided a method with which to explore the transitional states between reconsolidation-induction to extinction-induction conditions, allowing precise control over the number of times that the prediction of the US by the CS failed. Gradually increasing the number of CS presentations 'dose-dependently' resulted in a decrease in the freezing response measured at testing. BLA CaN level was negatively correlated with the percent of time spent freezing during the last CS presentation, with CaN levels being significantly increased only in the 10CS group. Enhancing NMDAR activity by systemic injection of DCS in animals receiving 7 CS presentations resulted in a behavioural (complete extinction) and molecular (high BLA CaN) pattern similar to that seen in the 10 CS group, indicating that the dominant memory process induced by the 7CS presentations is extinction. In marked contrast to the 1CS group, the 4CS group was, however, completely insensitive to manipulations of the NMDAR, with MK-801 and DCS injected groups showing a very similar level of freezing to the saline group. This striking finding strongly suggests that the transitional state induced by 4 CSs engages neither reconsolidation nor extinction. Remarkably, while MK-801 completely blocked the increase in CaN by 10 CSs in the BLA and prevented the consolidation of extinction memory, and DCS induced an increase in CaN and enhanced extinction in the 7CS group, both drugs had no behavioural or molecular effect in the 4CS group. These data provide key evidence in support of the behavioural and molecular dissociation between the transitional states induced by exposure to 4 and 7 CS presentations.

At least three alternative models can be employed to describe the NMDAR-dependent transition from reconsolidation to extinction: (i) a gradual transition model, with the transitional states reflecting different degrees of engagement of one or the other process (Figure 7A); (ii) a step function transition model, with reconsolidation and extinction being mutually exclusive (Figure 7B) and (iii) a three phase transition model (Figure 7C). While the first two models fail to predict the lack of significant behavioural

or molecular effect of bidirectional manipulation of NMDAR activity in the 4CS group, the experimental results favor the three phase transition model. Hence, a single or few CS presentations (less than 4) will induce the labilization and subsequent reconsolidation of the original fear memory; further presentations of the CS (e.g. 4) cancels the lability of the original memory and prevents reconsolidation, but fails to engage extinction. Finally, a greater number of CS presentations (7-10 CS) gradually engages extinction. Interestingly, it has been demonstrated that a second CS presentation can nullify the protein synthesis inhibition sensitivity of a fear memory induced 1h earlier by the first CS presentation (Jarome et al., 2012). The proposed molecular mechanisms responsible for apparently cancelling or nullifying the labilization processes that is initiated by early presentation of the CS are totally unknown. The presentation of a second, third or fourth CS (in our procedure) could trigger an interfering mechanism that might terminate the ubiquitin-proteasome system (UPS)-dependent labilization of the original memory induced by the early CS presentation (Lee et al., 2008), perhaps by local activation of deubiquitinating enzymes (Hegde, 2010) that previously have been associated with UPS homeostasis regulation in synaptic terminals (Cartier et al., 2009). Taken together, these data strongly suggest that reconsolidation and extinction of a CS-fear memory are mutually exclusive and that some transitional states induced by increasing numbers of non-reinforced CS presentations engage neither mechanism, reflecting a transition or 'limbo' state between updating, or reconsolidation, and inhibition through extinction learning.

Our present results demonstrate that the increase in CaN levels in the basolateral amygdala is uniquely and causally related to extinction. We have shown that reconsolidation and extinction of the cued fear memory are mutually exclusive, having distinct molecular characteristics, and we have also revealed that there is a novel insensitive, or 'limbo', state of the original memory that separates these processes.

Author Contributions

E.M. designed and conducted the experiments, with assistance from Z.Y.G. for the DCS-CaN study, and D.T. for the CaN knockdown study. E.M. prepared the figures and analyzed the data. E.M., A.L.M. and B.J.E. wrote the manuscript.

REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic acids research 25:3389-3402.

Baker JD, Azorlosa JL (1996) The NMDA antagonist MK-801 blocks the extinction of Pavlovian fear conditioning. Behav Neurosci 110:618-620.

Baumgartel K, Genoux D, Welzl H, Tweedie-Cullen RY, Koshibu K, Livingstone-Zatchej M, Mamie C, Mansuy IM (2008) Control of the establishment of aversive memory by calcineurin and Zif268. Nat Neurosci 11:572-578.

Blanchard RJ, Blanchard DC (1969) Crouching as an index of fear. Journal of comparative and physiological psychology 67:370-375.

Bouton ME (1991) Context and retrieval in extinction and in other examples of interference in simple associative learning. In: Current Topics in Animal Learning: Brain, Emotion and Cognition. (Dachowski L, Flaherty CF, eds). Hillsdale, NJ: Lawrence Erlbaum Associates.

Campeau S, Davis M (1995) Involvement of the central nucleus and basolateral complex of the amygdala in fear conditioning measured with fear-potentiated startle in rats trained concurrently with auditory and visual conditioned stimuli. In: The Journal of Neuroscience, pp 2301-2311.

Cartier AE, Djakovic SN, Salehi A, Wilson SM, Masliah E, Patrick GN (2009) Regulation of synaptic structure by ubiquitin C-terminal hydrolase L1. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:7857-7868.

Davis HP, Squire LR (1984) Protein synthesis and memory: a review. In: Psychological Bulletin, pp 518-559.

de la Fuente V, Freudenthal R, Romano A (2011) Reconsolidation or extinction: transcription factor switch in the determination of memory course after retrieval. The Journal of Neuroscience 31:5562-5573.

Dunn LT, Everitt BJ (1988) Double dissociations of the effects of amygdala and insular cortex lesions on conditioned taste aversion, passive avoidance, and neophobia in the rat using the excitotoxin ibotenic acid. Behav Neurosci 102:3-23.

Duvarci S, Nader K, LeDoux JE (2005) Activation of extracellular signal-regulated kinase- mitogen-activated protein kinase cascade in the amygdala is required for memory reconsolidation of auditory fear conditioning. The European journal of neuroscience 21:283-289.

Eisenberg M, Kobilo T, Berman DE, Dudai Y (2003) Stability of retrieved memory: inverse correlation with trace dominance. Science 301:1102-1104.

Gale GD, Anagnostaras SG, Godsil BP, Mitchell S, Nozawa T, Sage JR, Wiltgen B, Fanselow MS (2004) Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:3810-3815.

Garver TD, Kincaid RL, Conn RA, Billingsley ML (1999) Reduction of calcineurin activity in brain by antisense oligonucleotides leads to persistent phosphorylation of tau protein at Thr181 and Thr231. Molecular pharmacology 55:632-641.

Hegde AN (2010) The ubiquitin-proteasome pathway and synaptic plasticity. Learn Mem 17:314-327.

Herry C, Trifilieff P, Micheau J, Luthi A, Mons N (2006) Extinction of auditory fear conditioning requires MAPK/ERK activation in the basolateral amygdala. The European journal of neuroscience 24:261-269.

Herry C, Ciocchi S, Senn V, Demmou L, Muller C, Luthi A (2008) Switching on and off fear by distinct neuronal circuits. Nature 454:600-606.

Ikegami S, Inokuchi K (2000) Antisense DNA against calcineurin facilitates memory in contextual fear conditioning by lowering the threshold for hippocampal long-term potentiation induction. Neuroscience 98:637-646.

Ikegami S, Kato A, Kudo Y, Kuno T, Ozawa F, Inokuchi K (1996) A facilitatory effect on the induction of long-term potentiation in vivo by chronic administration of antisense oligodeoxynucleotides against catalytic subunits of calcineurin. Brain research Molecular brain research 41:183-191.

Jarome TJ, Kwapis JL, Werner CT, Parsons RG, Gafford GM, Helmstetter FJ (2012) The timing of multiple retrieval events can alter GluR1 phosphorylation and the requirement for protein synthesis in fear memory reconsolidation. Learn Mem 19:300-306.

Laurent V, Marchand AR, Westbrook RF (2008) The basolateral amygdala is necessary for learning but not relearning extinction of context conditioned fear. Learn Mem 15:304-314.

LeDoux JE (2000) Emotion circuits in the brain. Annual review of neuroscience 23:155-184.

Lee JLC, Everitt BJ, Thomas KL (2004) Independent cellular processes for hippocampal memory consolidation and reconsolidation. In: Science, pp 839-843.

Lee JLC, Milton AL, Everitt BJ (2006) Reconsolidation and extinction of conditioned fear: inhibition and potentiation. In: The Journal of Neuroscience, pp 10051-10056.

Lee SH, Choi JH, Lee N, Lee HR, Kim JI, Yu NK, Choi SL, Lee SH, Kim H, Kaang BK (2008) Synaptic protein degradation underlies destabilization of retrieved fear memory. Science 319:1253-1256.

Lin C-H, Yeh S-H, Leu T-H, Chang W-C, Wang S-T, Gean P-W (2003) Identification of calcineurin as a key signal in the extinction of fear memory. The Journal of Neuroscience 23:1574-1579.

Malleret G, Haditsch U, Genoux D, Jones MW, Bliss TV, Vanhoose AM, Weitlauf C, Kandel ER, Winder DG, Mansuy IM (2001) Inducible and reversible enhancement of learning, memory, and long-term potentiation by genetic inhibition of calcineurin. Cell 104:675-686.

Mao SC, Hsiao YH, Gean PW (2006) Extinction training in conjunction with a partial agonist of the glycine site on the NMDA receptor erases memory trace. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:8892-8899.

Milton AL, Merlo E, Ratano P, Gregory BL, Dumbreck JK, Everitt BJ (2013) Double dissociation of the requirement for GluN2B- and GluN2A-containing NMDA receptors in the destabilization and restabilization of a reconsolidating memory. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:1109-1115.

Miserendino MJD, Sananes CB, Melia KR, Davis M (1990) Blocking of acquisition but not expression of conditioned fear-potentiated startle by NMDA antagonists in the amygdala. In: Nature, pp 716-718.

Mulkey RM, Endo S, Shenolikar S, Malenka RC (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature 369:486-488.

Nader K, Schafe GE, LeDoux JE (2000) Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. In: Nature, pp 722-726.

Pavlov IP (1927) Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex. London: Oxford University Press.

Paxinos G, Watson C (2004) The Rat Brain in Stereotaxic Coordinates, 6th Edition, Academic Press.

Pedreira ME, Maldonado H (2003) Protein synthesis subserves reconsolidation or extinction depending on reminder duration. Neuron 38:863-869.

Suzuki A, Josselyn SA, Frankland PW, Masushige S, Silva AJ, Kida S (2004) Memory reconsolidation and extinction have distinct temporal and biochemical signatures. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:4787-4795.

Sweatt JD (2004) Mitogen-activated protein kinases in synaptic plasticity and memory. Current opinion in neurobiology 14:311-317.

Vianna MR, Szapiro G, McGaugh JL, Medina JH, Izquierdo I (2001) Retrieval of memory for fear-motivated training initiates extinction requiring protein synthesis in the rat hippocampus. Proceedings of the National Academy of Sciences of the United States of America 98:12251-12254.

Walker DL, Ressler KJ, Lu KT, Davis M (2002) Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats. In: The Journal of Neuroscience, pp 2343-2351.

FIGURE LEGENDS

Figure 1: BLA pERK1/2 is increased in reconsolidation and extinction, while CaN levels are only upregulated after extinction. (A). At training rats received two CS-US pairings. Twenty four hours later the animals were re-exposed to 1 or 10 CS to reactivate or extinguish the fear memory (n = 8 per group). The following day at the retention test all the animals were presented with 1 CS. (B). Twenty four hours after training the animals were injected IP with either saline (n = 4) or MK-801 (0.1 mg/Kg, n = 8) solution 30 minutes before being re-exposed to 1 CS. At test (LTM-TS), fear memory was measured by presentation of 1 CS. During training there was an effect of CS ($F_{(1, 10)} = 73.01$, $p < 0.001$, $\eta^2 = 0.88$), with no effect of group ($F_{(1, 10)} = 2.57$, $p = 0.14$). At reactivation, memory retention was evident since the animals froze significantly more to the fear CS ($F_{(1, 10)} = 25.63$, $p < 0.001$, $\eta^2 = 0.72$), with no effect of group ($F_{(1, 10)} = 2.19$, $p = 0.17$). At LTM-TS the MK-801 group showed a freezing response that was significantly lower than the saline group ($F_{(1, 10)} = 5.13$, $p < 0.05$, $\eta^2 = 0.34$) indicating the amnesic effect of MK-801 over memory reconsolidation. (C) The same behavioural procedure as in A, but with the inclusion of a non-reactivated control group (Ctr) that was trained identically to the 1CS and 10CS groups but during the re-exposure session remained in the home cage. Twenty (pERK1/2) or 60 (CaN) minutes after re-exposure, or straight from the home cage (Ctr groups), animals were sacrificed and the cytoplasmic protein extracts from the BLA obtained (all groups n = 8). (D) and (E). Representative western blot results and analysis showing the cytosolic levels of pERK1/2 (D) or CaN (E) after 1 or 10 CS presentations. * $p<0.05$, ** $p<0.01$.

Figure 2: Schematic representation of the brain showing the placement of the infusion sites in animals in experiments described in Figure 3, 1 and 10 CS groups. All injections sites were within the BLA. Open circles: Scr injections. Close circles: CaN ASO injections. Coordinates are given from bregma. This figure was modified, with permission, from Paxinos and Watson (Paxinos and Watson, 2004).

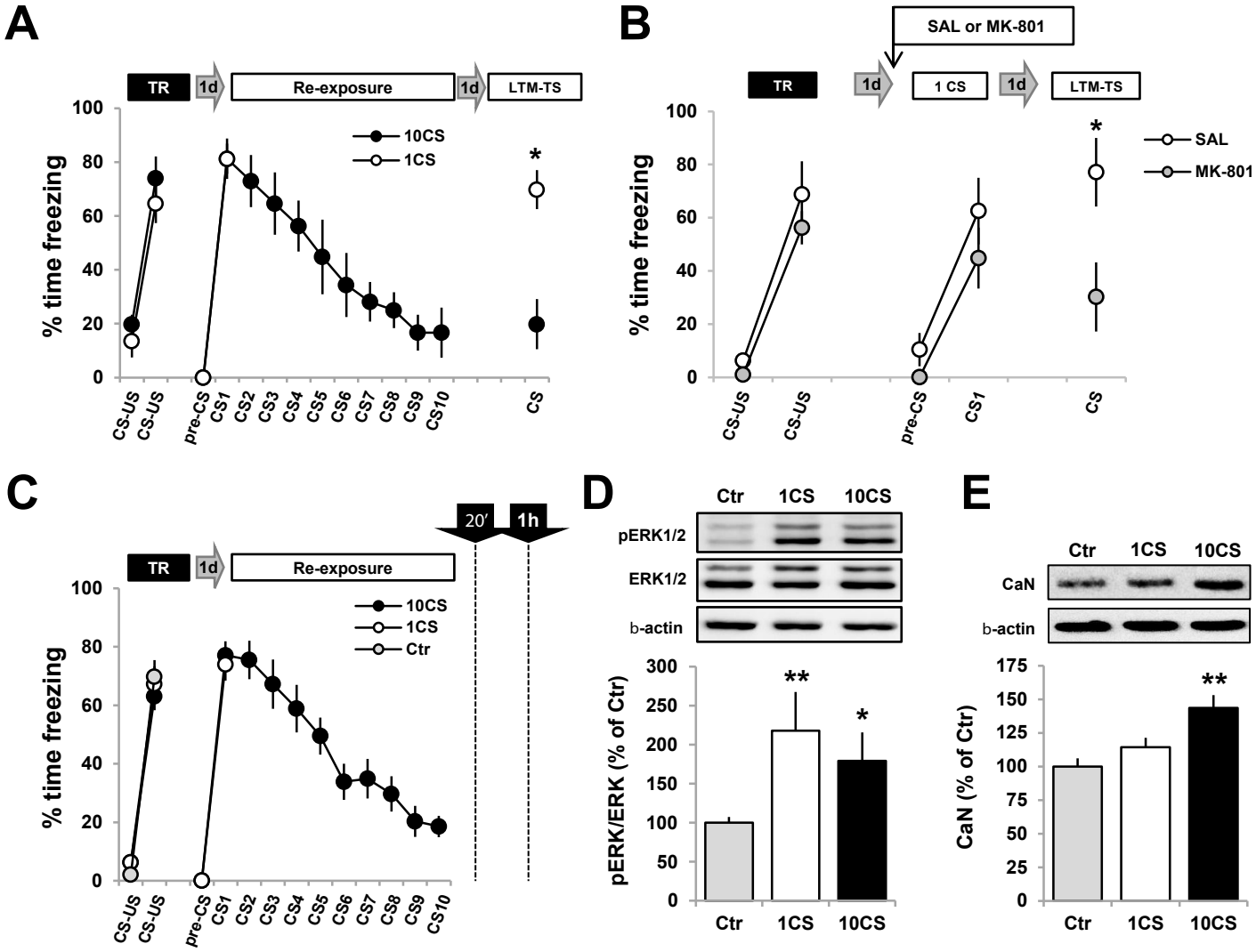
Figure 3: CaN increase induced by 10 CS is required for consolidation of the fear extinction memory. (A). Animals with fully consolidated fear memory received microinfusions of CaN antisense (ASO) or scrambled (Scr) oligodeoxynucleotide (ODN) sequences in the BLA (see Figure S2) 2.5 hours before the presentation of 1 (n = 10 per group) or 10 CS (Scr n = 10, ASO n = 8). Four days later all groups were tested for long-term fear or extinction memory retention by the presentation of one CS. * $p<0.005$. (B). Animals with fully consolidated fear memory were microinfused with ASO (n = 5) or Scr (n = 4) 2.5 hours before 10 CS presentations. One hour after the 10 CS they were sacrificed and the BLA cytoplasmic extract obtained. A representative result of the western blot analysis along with the quantification of CaN levels in the BLA is shown. * $p<0.05$.

Figure 4: BLA CaN levels are negatively correlated with the level of conditioned freezing. (A). Animals with a fully consolidated fear memory were re-exposed to 1, 4, 7 or 10 CS and tested 24 h later (n = 8 per group). * $p<0.05$ vs. 1CS group. (B). Level of freezing at the last CS of the re-exposure session vs. level of freezing at test (LTM-TS). (C). Identical experimental procedure as in a, but animals were sacrificed 1 hour after the presentation of 1 (n = 12), 4 (n = 7), 7 (n = 8) or 10 (n = 12) CS along with a control group (Ctr, n = 16) that received training the day before and was sacrificed directly from the home cage. Left: level of freezing during the training and CS presentation sessions. Right: representative western blot and level of cytosolic CaN in the BLA (Ctr group not shown). ** $p<0.01$. (D). Correlation analysis between the level of CaN in the BLA and the % time freezing at the last CS of the re-exposure session. Correlations were as follows: number of CS vs freezing level ($r_S(39)= -0.734$, $p<0.01$); number of CSs vs CaN level ($r_S(39)= 0.518$, $p<0.01$); freezing level vs CaN level ($r_S(39)= -0.345$, $p<0.05$).

Figure 5: NMDAR agonist and antagonist treatments differentially affect the behavioural transition between reconsolidation and extinction. (A). Experimental design. One day after training, the rats received an intraperitoneal injection of the NMDAR partial agonist DCS (15 mg/Kg) or the antagonist MK-801 (0.1 mg/Kg). One quarter of each group received either 1 (B), 4 (C), 7 (D) or 10 (E) CS presentations (line graphs). Twenty four hours later freezing behaviour was measured in all groups by presentation of 1CS (bar graphs). MK-801 groups: 1CS, n = 12; 4CS, n = 9; 7CS, n = 9 and 10CS, n = 11. DCS groups: 1CS, n = 7; 4CS, n = 8; 7CS, n = 8 and 10CS, n = 8. * $p<0.05$, ** $p<0.01$.

Figure 6: NMDAR agonist and antagonist treatments modulate levels of CaN in the BLA induced by increasing numbers of CS presentations. The procedure was as described in Figure 4, but all animals were sacrificed 1 hour after the CS re-exposure session to quantify the levels of CaN in the BLA. As control groups, animals were trained, and 24h later injected with MK-801 (A) or DCS (B) and sacrificed 90 minutes after from the home cage. Representative western blot results are shown for each experiment. Bar graphs: level of CaN in the BLA of animals receiving MK-801 (A) or DCS (B) 30 minutes before being presented with 1, 4, 7 or 10 CS. MK-801 groups: Ctr = 7, 1CS, n = 7; 4CS, n = 7; 7CS, n = 8 and 10CS, n = 8. DCS groups: n = 10 each. * $p<0.05$.

Figure 7: Models of the transition from reconsolidation to extinction of fear memories. (A). Gradual model. (B). Step function model. (C). Three phase model. R: reconsolidation process. E: extinction process. Y axis: sensitivity to NMDAR activity manipulations. X axis: number of CS presentations.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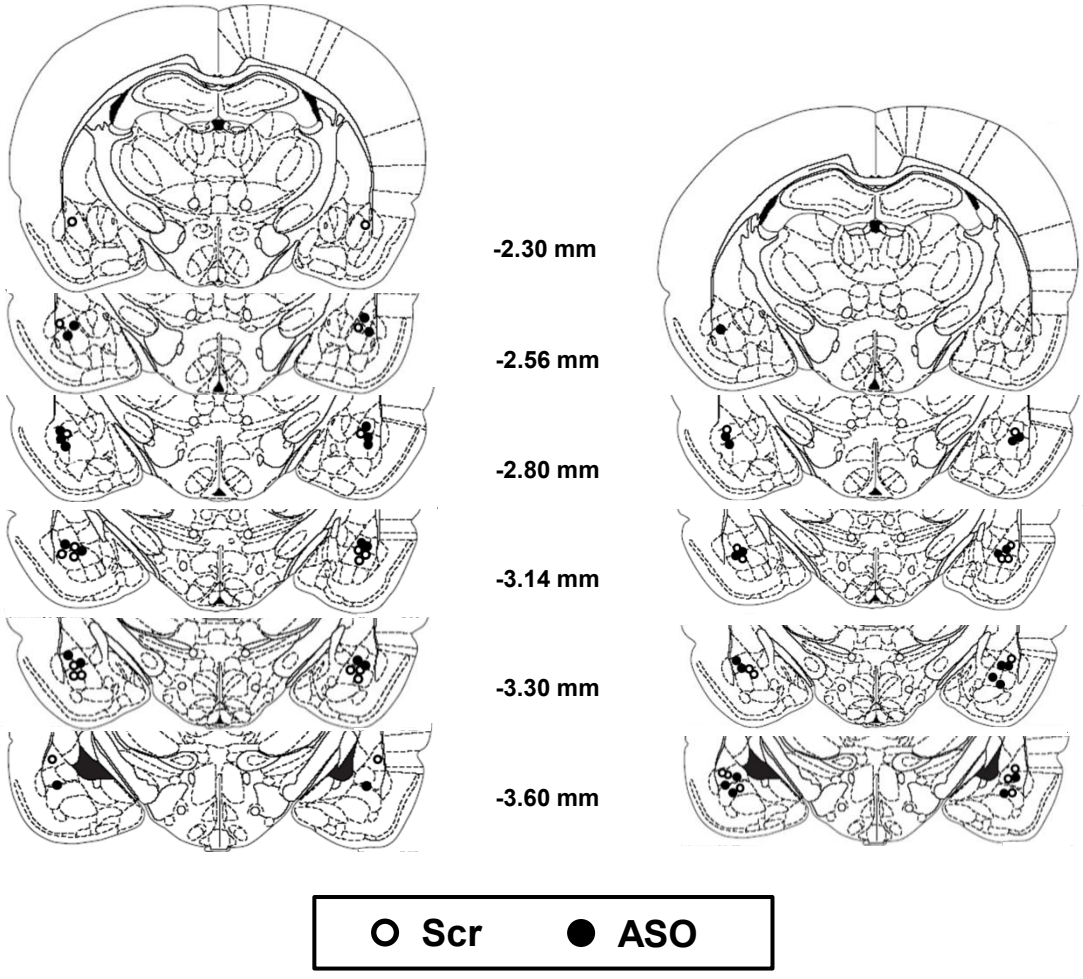
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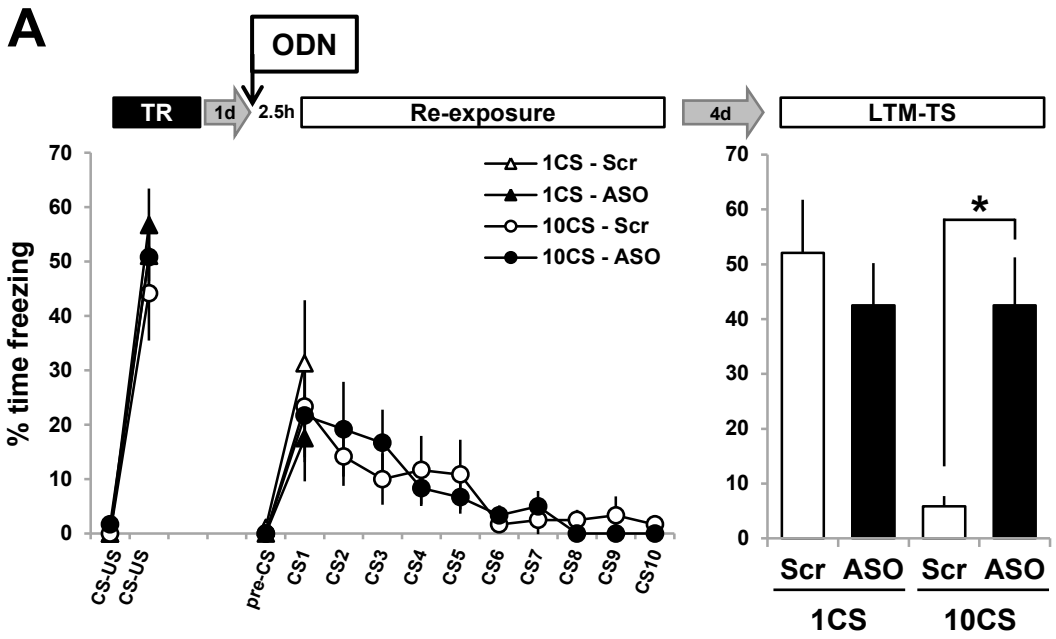
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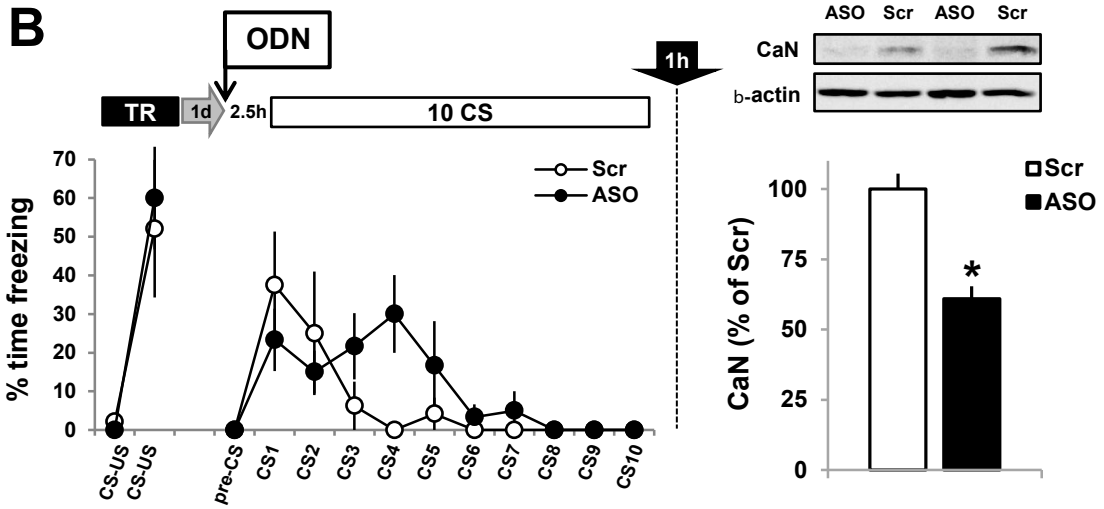
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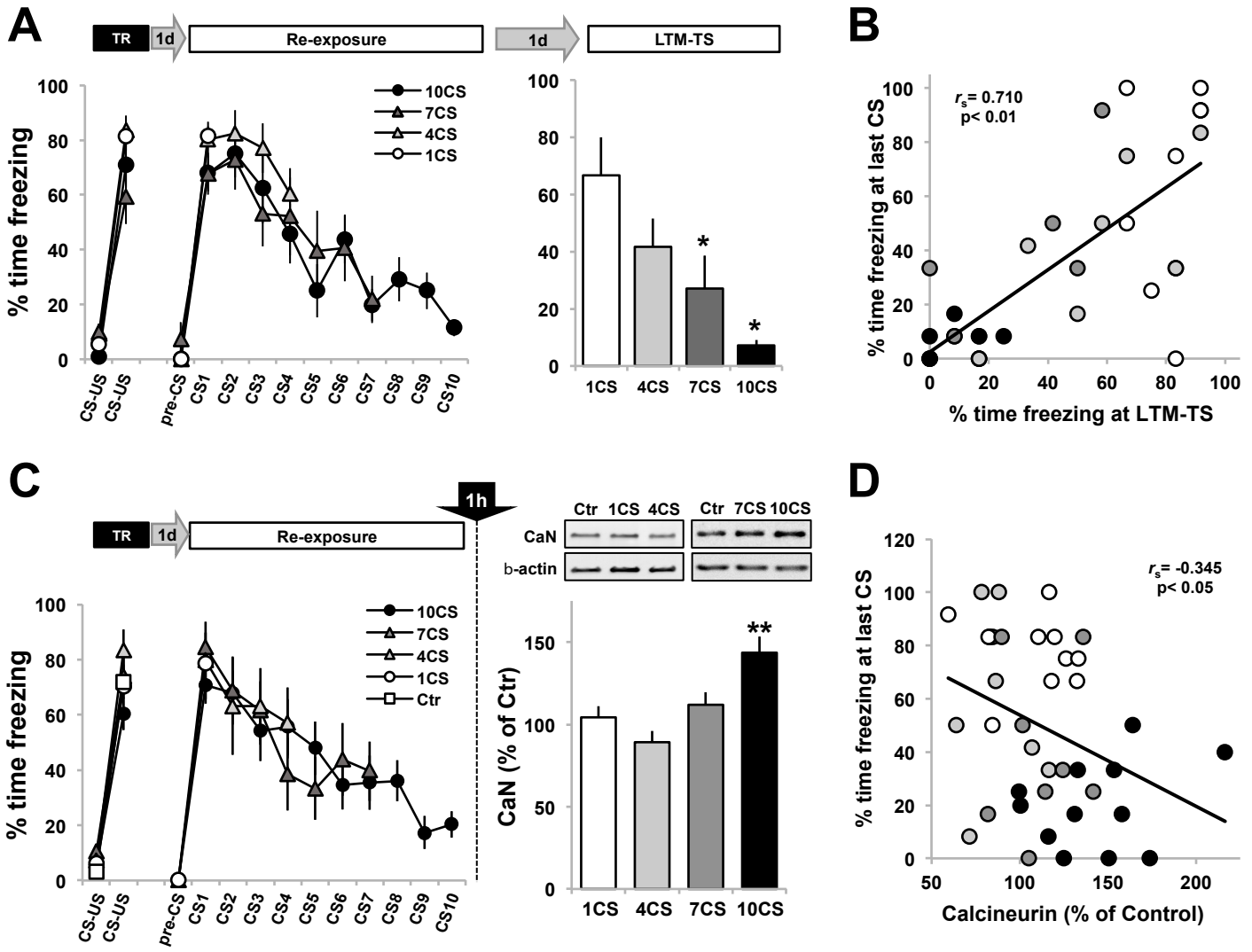
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p< 0.01
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C
1h
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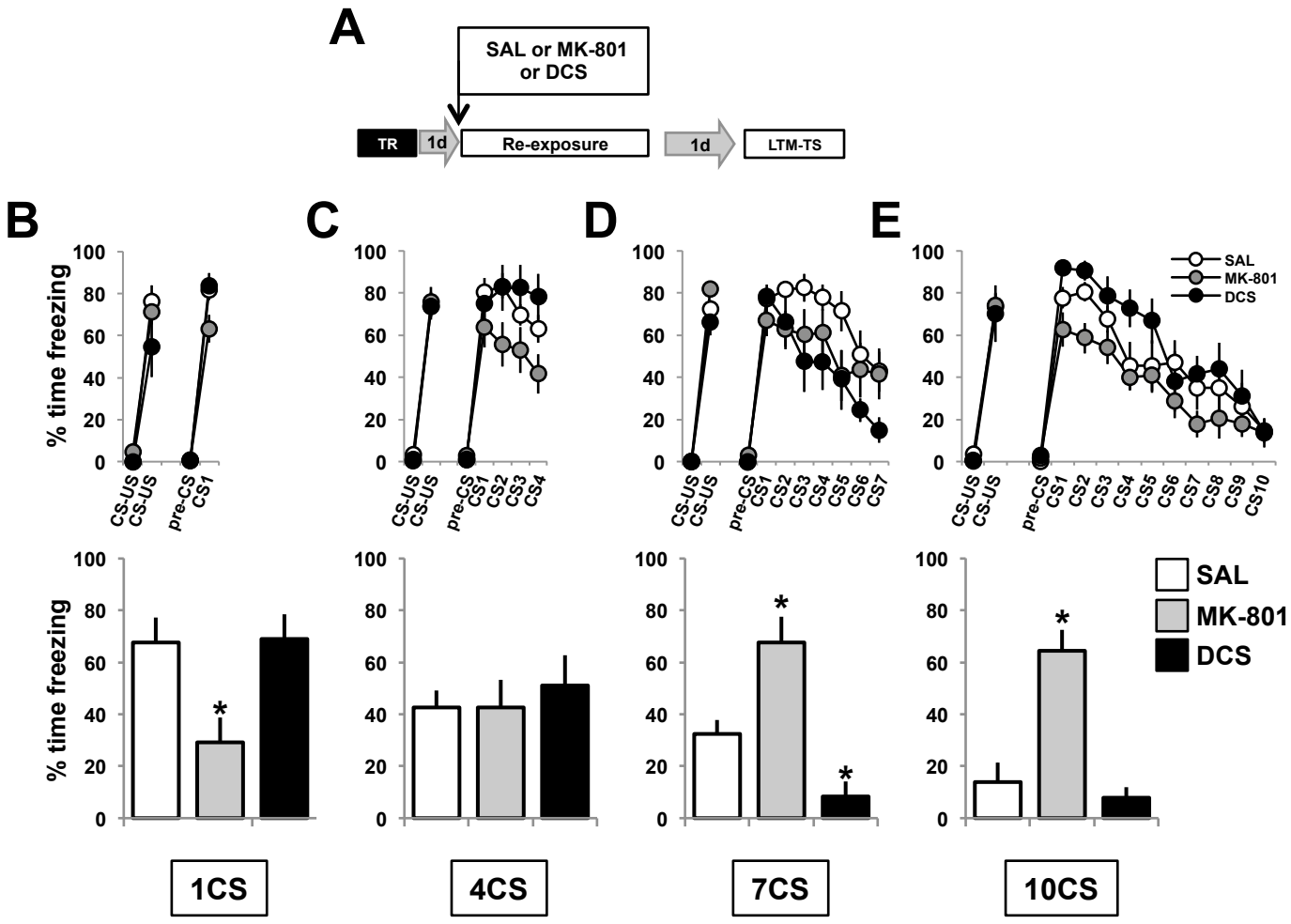
Calcineurin (% of Control)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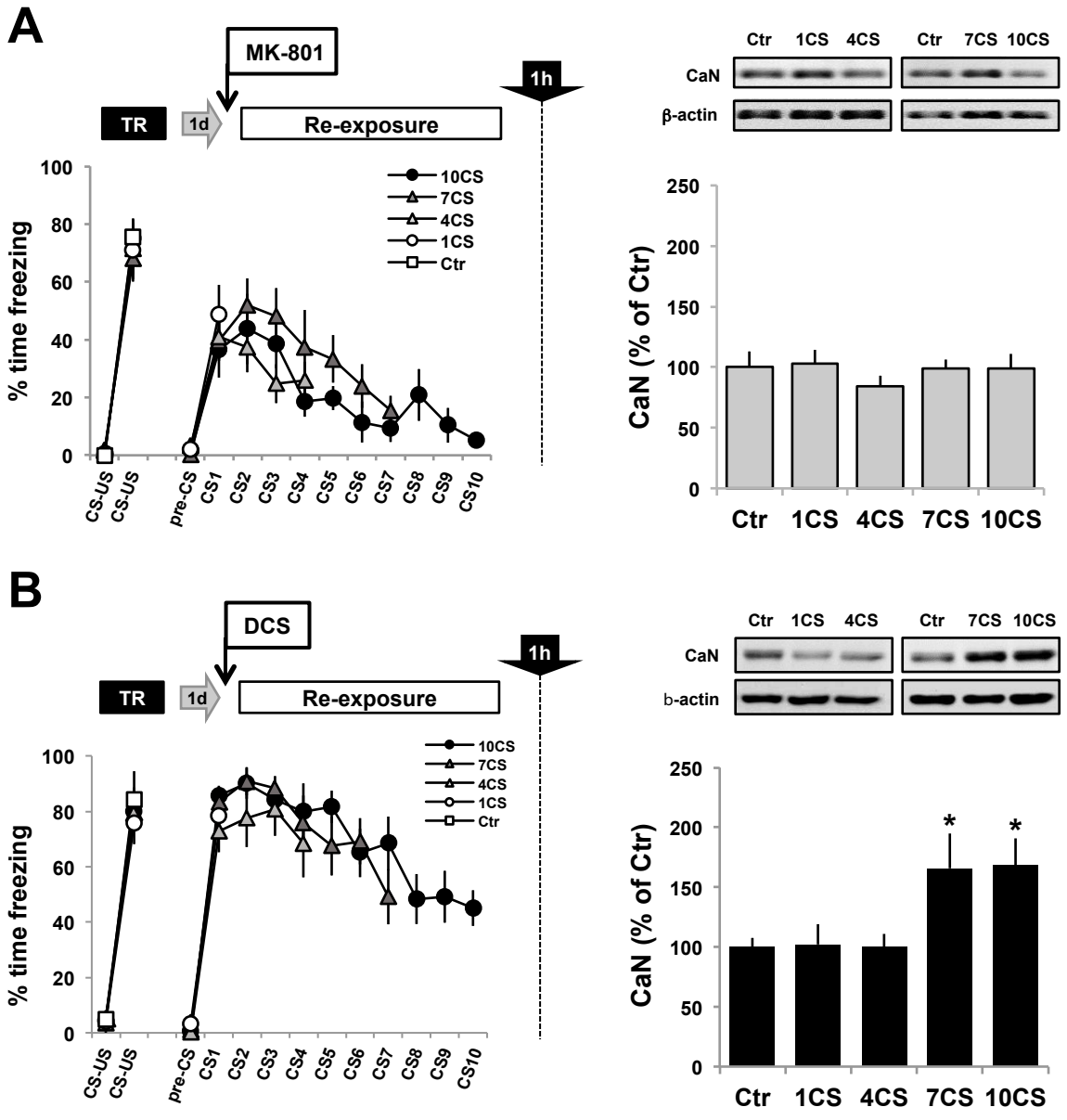
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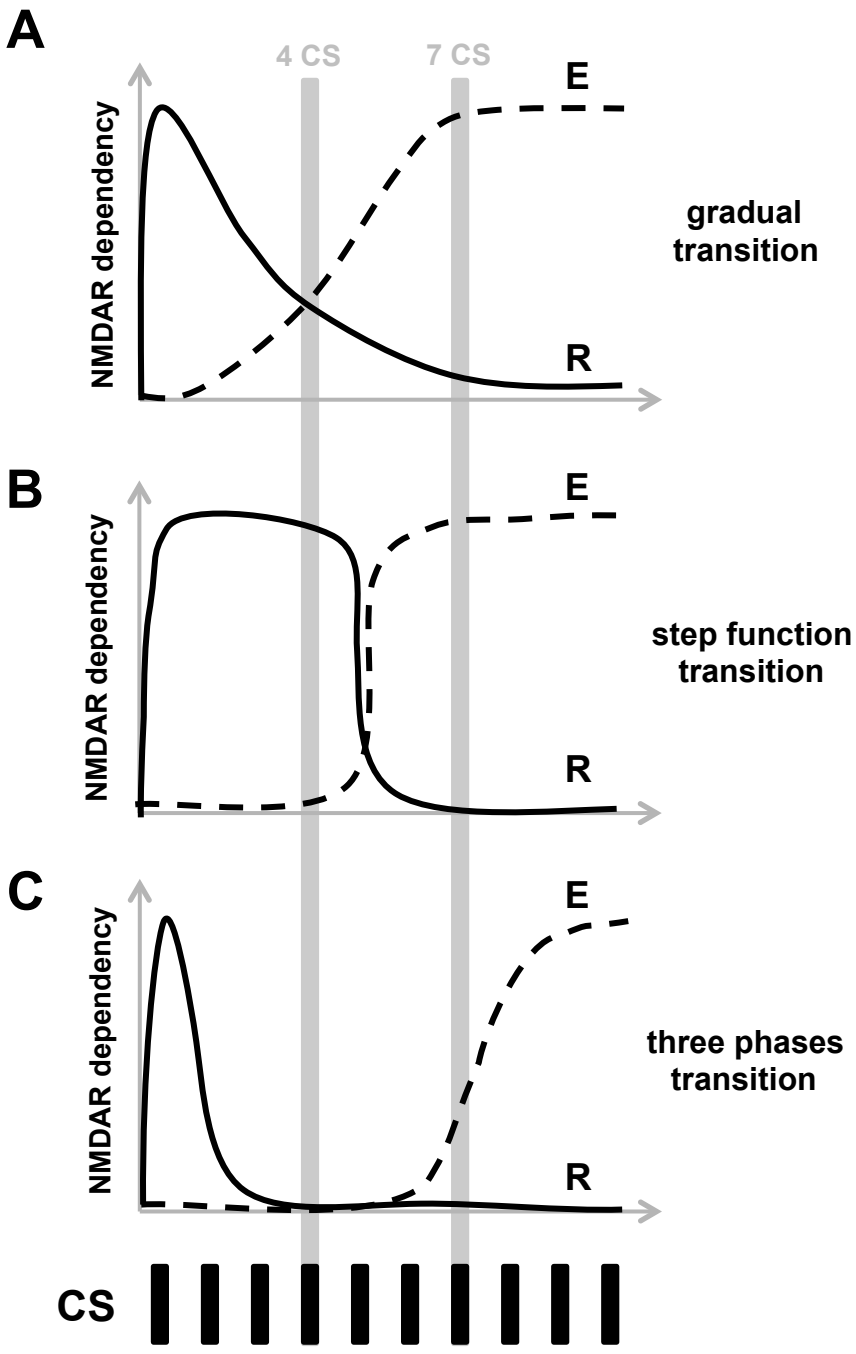
CS1
CS2
CS3
CS4
CS5
CS6
CS7
CS8
CS9
CS10
Ctr 1CS 4CS Ctr 7CS 10CS
CaN
β-actin
CaN (% of Ctr)
Ctr 1CS 4CS 7CS 10CS
B
DCS
TR
1d
Re-exposure
1h
10CS
7CS
4CS
1CS
Ctr
% time freezing
CS-US
CS-US
pre-CS
CS1
CS2
CS3
CS4
CS5
CS6
CS7
CS8
CS9
CS10
Ctr 1CS 4CS Ctr 7CS 10CS
CaN
b-actin
CaN (% of Ctr)
*
*
Ctr 1CS 4CS 7CS 10CS


A
4 CS
7 CS
E
NMDAR dependency
R
gradual
transition
B
E
NMDAR dependency
R
step function
transition
C
E
NMDAR dependency
R
three phases
transition
CS